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Identification of Putative Photoreceptor Cells in the Siphon of a Clam, *Ruditapes philippinarum*

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ABSTRACT—A clam, *Ruditapes philippinarum* responds to light by siphonal retraction and valve adduction. Sensitivity to light was seen widely diffused on the siphon, so attempts to identify possible photoreceptor cells were made in the distal portion of the siphon which is most sensitive. Histological investigations by light- and electron-microscopy revealed microvilli arising from the epithelial cells of the outer and inner surfaces of the siphon. Immunohistochemical experiments using anti-squid-rhodopsin serum in conjunction with FITC or streptavidin-biotin revealed specific binding of the anti-serum to the microvillar layer of the outer and inner epithelial cells. It is therefore suggested that the epithelial cells may contain a visual pigment in the microvilli and so function as a primitive photoreceptor.

INTRODUCTION

The phylum Mollusca includes a wide variety of forms, which have evolved a correspondingly diverse range of visual organs, from 'simple' eyes comprising relatively few cells to sophisticated organs with a lens, pupil and a complex retina [14]. The cephalopods (octopuses and squids), for examples catch mobile prey and, relying heavily on vision, have developed eyes comparable to those of vertebrates. Many gastropods, which generally move about slowly on the sea bed have a relatively simple 'eye spot' [6–8]. The bivalves are filter feeders, which are usually attached to the substrate and except for Pectinidae [1] and Cardiidae [2, 22] generally lack sophisticated photoreceptive organs. Instead, they have a primitive photoreceptive system consisting of scattered photoreceptor cells which have been demonstrated indirectly by recording light-activated impulses from the pallial nerves in *Spisula, Mya* and *Venus* ([10], for review see [24]).

It is clear from preliminary behavioral observations that many bivalves of the family Veneridae respond to light but the organs of photoreception have yet to be identified. *Ruditapes phillipinarum*, a common venerid in Japan, shows siphonal retraction and valve adduction in response to either an increase or decrease in background light. Preliminary investigations on *Ruditapes*, with a small light spot of about 1 mm in diameter, indicated that photosensitivity was widely distributed over the siphon, suggesting that photoreceptor cells are scattered throughout the regions. The aim of the present investigations was to identify the photoreceptor cells in the most sensitive region: the distal portion of the siphon.

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MATERIALS AND METHODS

Small specimens of *Ruditapes philippinarum* (Adam & Reeve, 1850), were collected near the Ushimado Marine Laboratory or purchased from fish shops. Prior to the experiments, the clams were dark-adapted or kept under dim red light for more than 4 hr. The tip of the siphon was then removed from the animals and used for the experiments described below.

Microscopy

For light-microscopical observations, isolated siphons were fixed in Bouin's fluid for a day, dehydrated through a graded series of ethanols and embedded in paraffin wax. Sections of 4 μm thick were stained with hematoxylin-eosin.

For electron-microscopical observations, isolated siphons were fixed with 1% glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH 7.4) and 0.4 M sucrose for about 4 hr at room temperature. The material was post-fixed with 1% osmium tetroxide and 1% potassium ferrocyanide in 0.1 M sodium cacodylate (pH 7.4) and 0.4 M NaCl. The samples were dehydrated through a graded series of ethanols and embedded in epoxy-resin (TAAB Laboratories). Ultrathin sections were stained with 1% alcoholic uranyl acetate and then 0.1% lead citrate and examined with a Hitachi H-500H electron microscope.

Immunohistochemistry

To investigate whether or not the siphons of *Ruditapes* contain a visual pigment (rhodopsin), an anti-rhodopsin serum was prepared. A procedure to purify a *Ruditapes* rhodopsin has not yet been estabilished. Instead, a squid (*Todarodes pacificus*) rhodopsin was used for immunization.

The anti-squid rhodopsin serum was prepared as follows. The *Todarodes* rhodopsin was extracted and purified according to the procedure reported by Nashima *et al* [16]. Rhodopsin obtained was further purified with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A rhodopsin band was collected and dissolved in phosphate-buffered saline. Immunization was carried out by injecting the dissolved sample containing rhodopsin (about 50

μg protein) into a mouse 3 times, every 3 weeks. Prepared mouse anti-squid rhodopsin serum recognized the rhodopsin bands in western blot analysis of *Todarodes* retina and immunohistochemically outer segments of photoreceptor cells of *Todarodes* retina.

Isolated siphons were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) containing 0.4 M sucrose under dim red light for more than 8 hr at 4°C. The siphons were cryoprotected by soaking in the same buffer solution containing 30% sucrose for 2 hr and then embedded in OCT compound (Miles Scientific, No. 4583) for freezing in liquid nitrogen. The frozen samples were sectioned at 5 μm or 15 μm with a cryotome (Bright, Model OTF) at −20°C.

Thicker sections of 15 μm were first treated with 0.1% Triton X-100 for 15 min and then with 0.02% glycine for 2 hr to block non-specific autofluorescence. The sections were primarily reacted with the anti-rhodopsin serum, diluted 200 times, for 2 hr at room temperature and secondarily with fluorescein isothiocyanate(FITC)-conjugated goat anti-mouse IgG for 3 hr at room temperature. For the control, the anti-rhodopsin serum was absorbed with purified *Todarodes* rhodopsin. Phosphate buffer (0.1M) was used in a series of experiments. Stained sections were observed by epifluorescence.

To avoid autofluorescence involved in the immunofluorescence technique, localization of rhodopsin was also investigated by using a modified avidin-biotin complex (ABC) immunohistochemical technique, i.e., Streptavidin-Biotin (SAB) method (Nichirei, Histofine, SAB-PO(M) kit). Thinner sections of 5 μm and the same anti-rhodopsin serum as that described above (but diluted 500 times with buffer) were used. A series of reactions were performed according to the kit protocol. Sections were counterstained with hematoxylin.

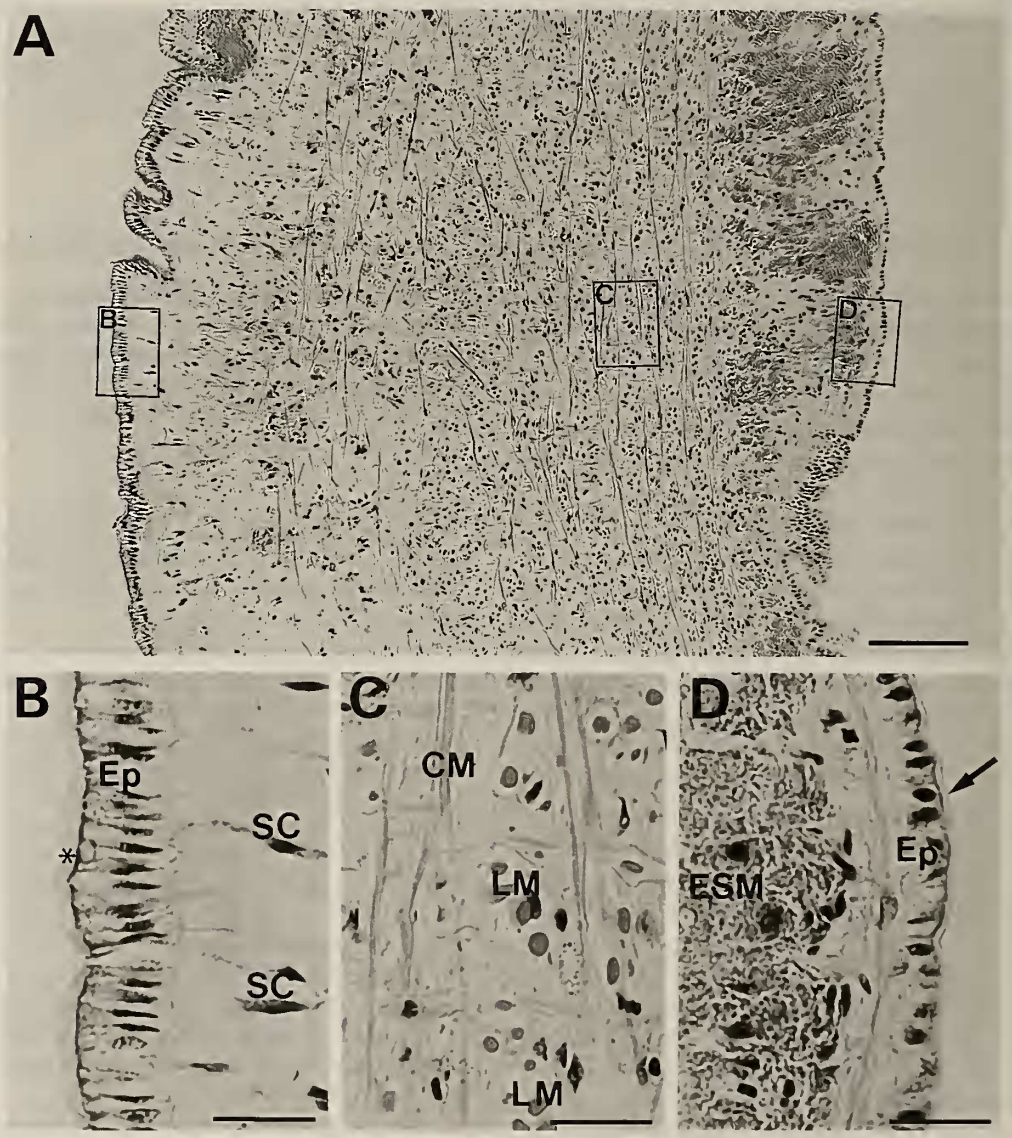

FIG. 1. Light micrographs of a transverse section through the siphonal wall. (A) Low power view. The left and right boundaries correspond to the outer and inner walls of the siphon, respectively. (B)-(D) Insets shown in (A) at higher magnification. (B) Outer epithelium. Note the translucent layer (asterisk) overlying the epithelium (Ep). Cells presumed to be sensory cells (SC) extend fine processes distally and proximally. (C) Muscle tissue of siphon, consisting of circular (CM) and longitudinal (LM) muscles. (D) Inner epithelium. Arrow indicates the outermost translucent layer, similar to that in B. Below the inner epithelium are aggregations of eosin sensitive matter (ESM). Scale bars 100 μm in A, 20 μm in B-D.

RESULTS

Light microscopical observations

The siphon of *Ruditapes* consists of inhalent and exhalent tubes fused with each other on one side, having a figure-8-like form in cross-section. Figure 1A shows a cross-sectional view through the siphonal wall. As magnified in Figure 1B, the extreme outer surface of the siphon is covered with a translucent layer (asterisk in B), which is stained with neither hematoxylin nor eosin, under which columnar cells with elliptical nuclei proximally are present. Cells presumed to be sensory cells lie below an outer epithelial layer (outer EPL) and extend fine processes distally towards the outer surface and proximally towards the central region of the siphonal wall. The central region is occupied with circular and longitudinal muscles which intermingle with each other (Fig. 1C). The inner wall of the siphon is also covered with a layer of cells somewhat thinner than that of the outer ones (Fig. 1D). The translucent layer is also seen along the surface of an inner epithelial layer (inner EPL). Additionally, there are large aggregations of material staining with eosin throughout the region below the inner EPL (Fig. 1A, D). It is emphasized that no structures (such as eye cup, reflecting tapetum and lens) featuring differentiated photoreceptor organs were observed. There were, at least light-microscopically, no apparent differences between the inhalent and exhalent siphons.

Immunohistochemical observations

To investigate the location of photoreceptive pigment, rhodopsin, the siphon was searched by using an anti-serum against squid rhodopsin. Greenish specific fluorescence due to conjugated FITC was observed as a layer along the free surface of the outer EPL (Fig. 2A). This fluorescence was not seen when anti-rhodopsin serum was absorbed by purified squid rhodopsin: only dim yellow autofluorescence remained along the line of cell bodies in the outer EPL (Fig. 2B).

The inner EPL also fluoresced on their free surface, although somewhat weaker than the outer EPL (Fig. 2C). In addition, intense fluorescence was detected throughout the region below the inner EPL (Fig. 2C). Fluorescence in both areas disappeared in the control and dim yellow autofluorescence remained along the line of cell bodies in the inner EPL (Fig. 2D).

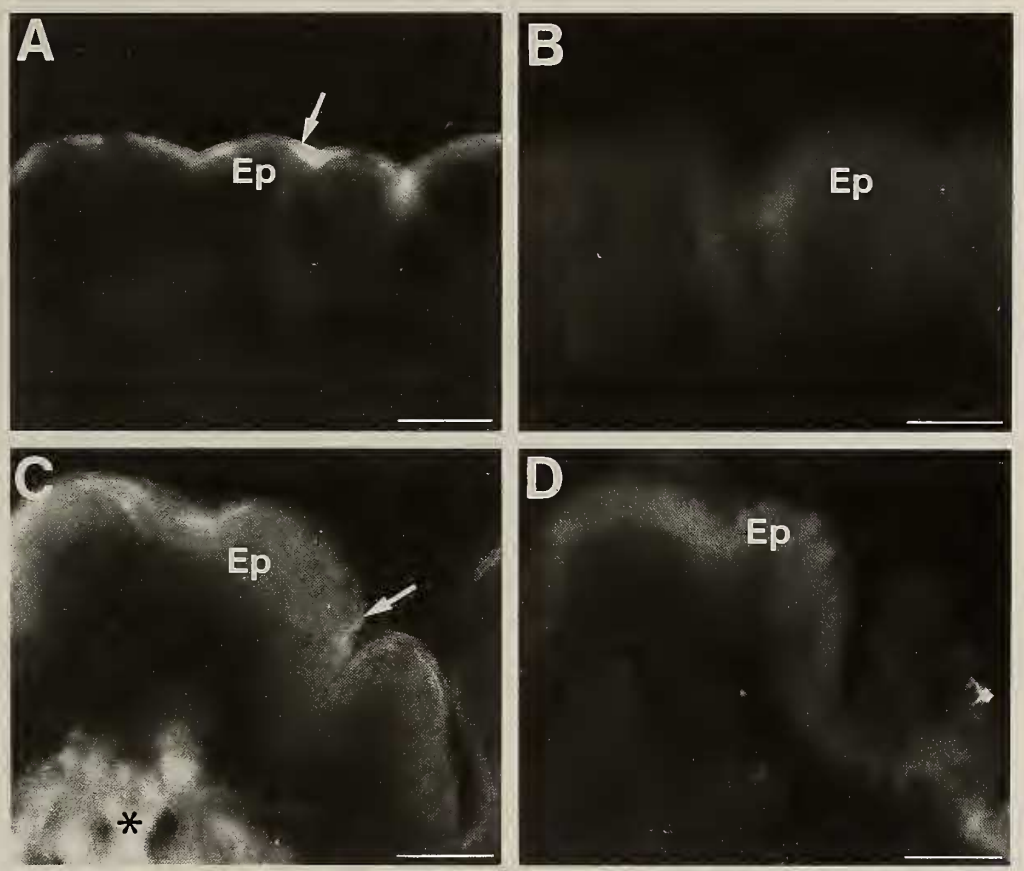

FIG. 2. Fluorescence micrographs of the epithelia of the siphon immunostained with anti-rhodopsin serum using the FITC method. (A) A fluorescing layer (arrow) along the free surface of the outer epithelium (Ep). (B) Control. Anti-rhodopsin serum was blocked by purified rhodopsin. Little fluorescence appears. (C) A fluorescing layer (arrow) along the free surface of the inner epithelium (Ep). Intense fluorescence (asterisk) was found throughout the region below the inner epithelium (cf. ESM in Fig. 1D), (D) Control of C, using anti-rhodopsin serum blocked by purified rhodopsin. Most fluorescence (cf. that indicated by the arrow and asterisk in C) disappeared leaving auto-fluorescence from the cell bodies of the inner epithelium. Scale bars, 20 μm.

To avoid the auto-fluorescence seen when using FITC, the anti-rhodopsin serum was also used in conjunction with the Streptavidin-Biotin (SAB) method. As was the case for FITC, binding of the antiserum was restricted to the extreme surface of the outer EPL (Fig. 3A) and lost in the control with the antiserum which was absorbed by purified squid rhodopsin (Fig. 3B). The inner EPL gave similar results (Fig. 3C, D). However, weaker specific binding than that of the inner EPL was found throughout the region just below the inner EPL (Fig. 3C), corresponding to the strong specific fluorescence observed with FITC (Fig. 2C).

Identification of immunoreactive cells

To identify the cells positive to anti-rhodopsin serum, the ultrastructure of the siphon was investigated (Fig. 4). As shown in the light-microscopical observations, the electron-microscopic observations reveal that the outer surface of the siphon is covered with epithelial cells (outer EPCs)(10–20 μm in length, 4–7 μm in width) which are anchored by connective tissue through a basement membrane (Fig. 4A). A nucleus occupies the base of each cell and a number of vesicles and mitochondria are present distally. Some cells have tightly packed electron-dense melanin-like granules. Numerous microvilli (3–5 μm in length, 50–100 nm in diameter) arise from the free surface of the outer EPCs, forming a layer which coincides in position with the translucent layer observed in the light microscope (Fig. 1).

The inner epithelial cells (inner EPCs) (3–10 μm in length, 4–7 μm in width) have less vesicles than the outer EPCs, but otherwise basically resemble the outer EPCs (Fig. 4B). The microvilli (0.5–3 μm in length, 50–100 nm in diameter) are also observed on the free surface of the inner EPCs. Beneath the layer of the connective tissue underlying the inner EPCs, cells with tightly packed granules (0.3–0.4 μm in diameter) are seen in groups (Fig. 4B). They correspond in position to the eosin-sensitive matters (ESM) in Figure 1D and will hereafter be referred to as 'granular cells'.

Although much less frequently, two other types of cell, are found among the outer and inner EPCs. One type has many 'cilia' on the free surface, and the other has shorter

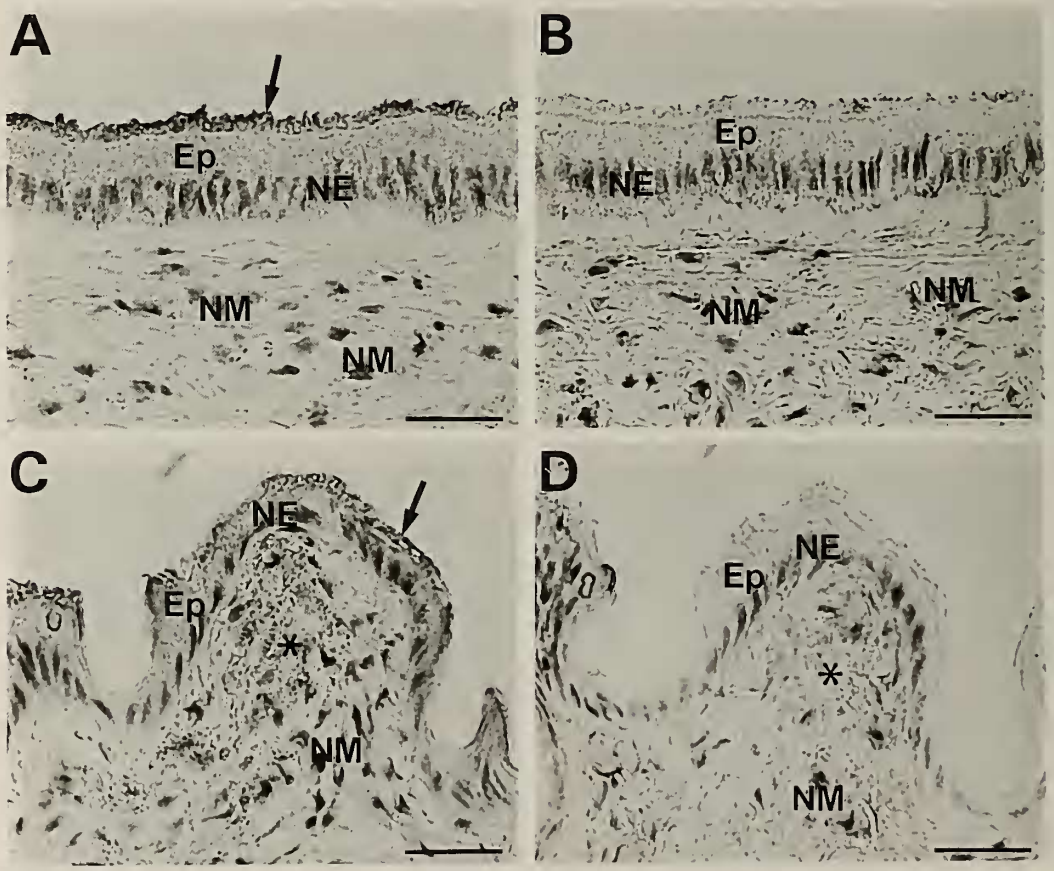

FIG. 3. Light micrographs of the outer (A, B) and inner (C, D) surfaces of the siphon immunostained with anti-rhodopsin serum using the SAB method, counterstained with hematoxylin. (A) A dark layer of positive staining (arrow) appears along the free surface of the outer epithelium (Ep). (B) Control. Anti-rhodopsin serum blocked by purified rhodopsin does not stain the free surface of the outer epithelium. The hematoxylin-stained nuclei of the outer epithelium (NE) and muscles (NM) remain unchanged between A and B. (C) The free surface of inner epithelium (Ep) stained with the anti-serum (arrow). Weaker binding (asterisk) appears throughout the region below the inner epithelium. (D) Control of C, using rhodopsin-blocked anti-serum. The nuclei (NE, NM) counter-stained with hematoxylin remain unchanged but the staining seen in C does not appear. Scale bars, 20 μm.

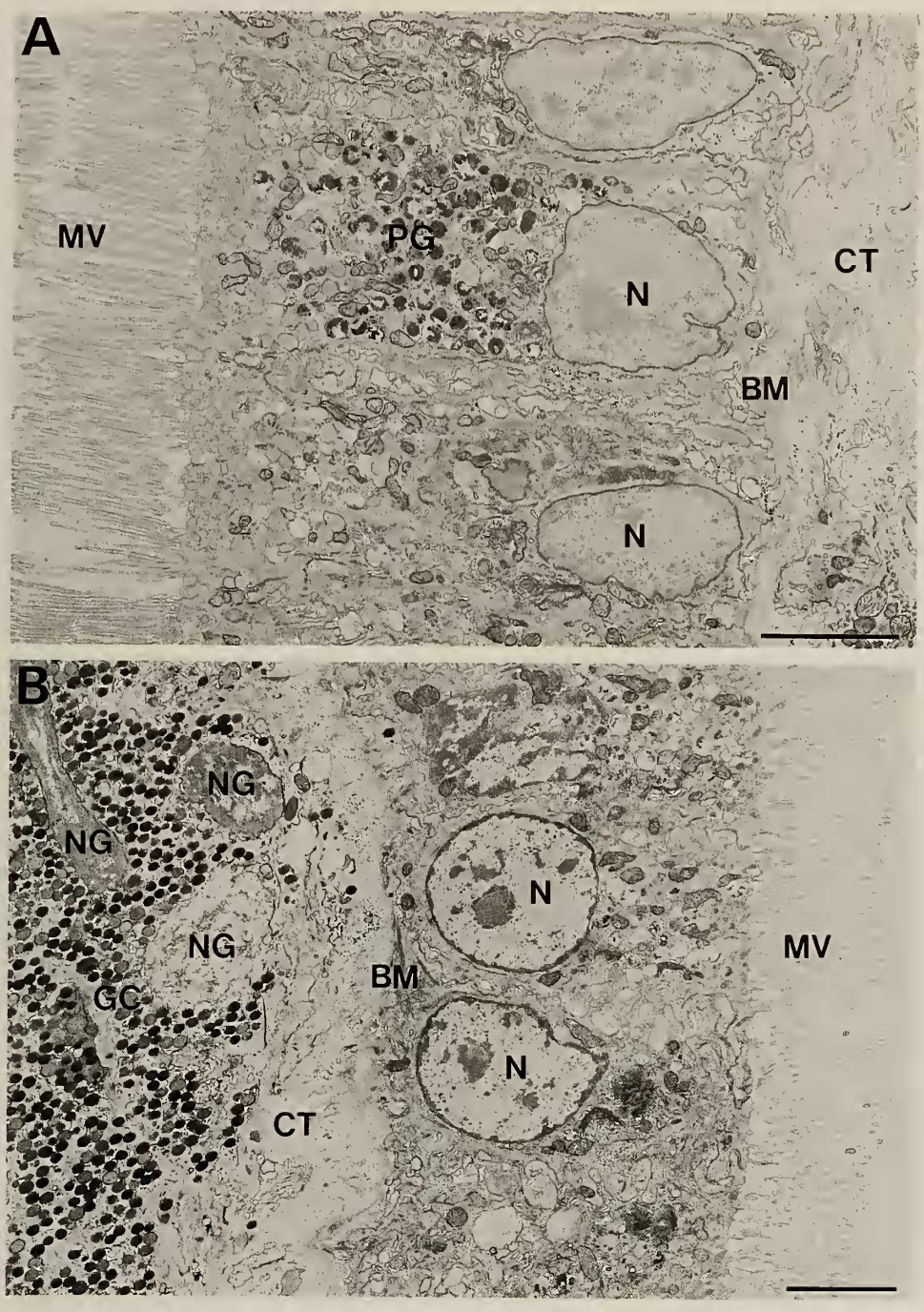

FIG. 4. Electron micrographs of the outer (A) and inner (B) walls of the siphon. Note the characteristic microvilli arising from the outer and inner EPCs, and the granular cells (GC) with numerous compacted granules. (Abbreviations) BM, basement membrane; CT, connective tissue; MV, microvilli; N, nuclei of epithelial cells; NG, nuclei of granular cells; PG, pigment granules. For explanations, see text. Scale bars, 3 μm.

microvilli, forming a concaved 'pit'-like structure distally (Fig. 5A). Cilia could not be found in the latter even in a series of continuous sections. Both 'ciliary' and 'pit' cells extend microtuble-containing fine processes distally between the outer and inner EPCs (Fig. 5B, C) and have nuclei proximally (not shown in Fig. 5). It is clear from their appearance and position that they correspond to the sensory cells in Figure 1B. The central region of the siphonal wall is mostly occupied by the circular and longitudinal muscles, with scattered nerve bundles and fine nerve processes (Fig. 5D). However, differentiated photoreceptor cells comparable to those in eyes were never found in the siphon of *Ruditapes*.

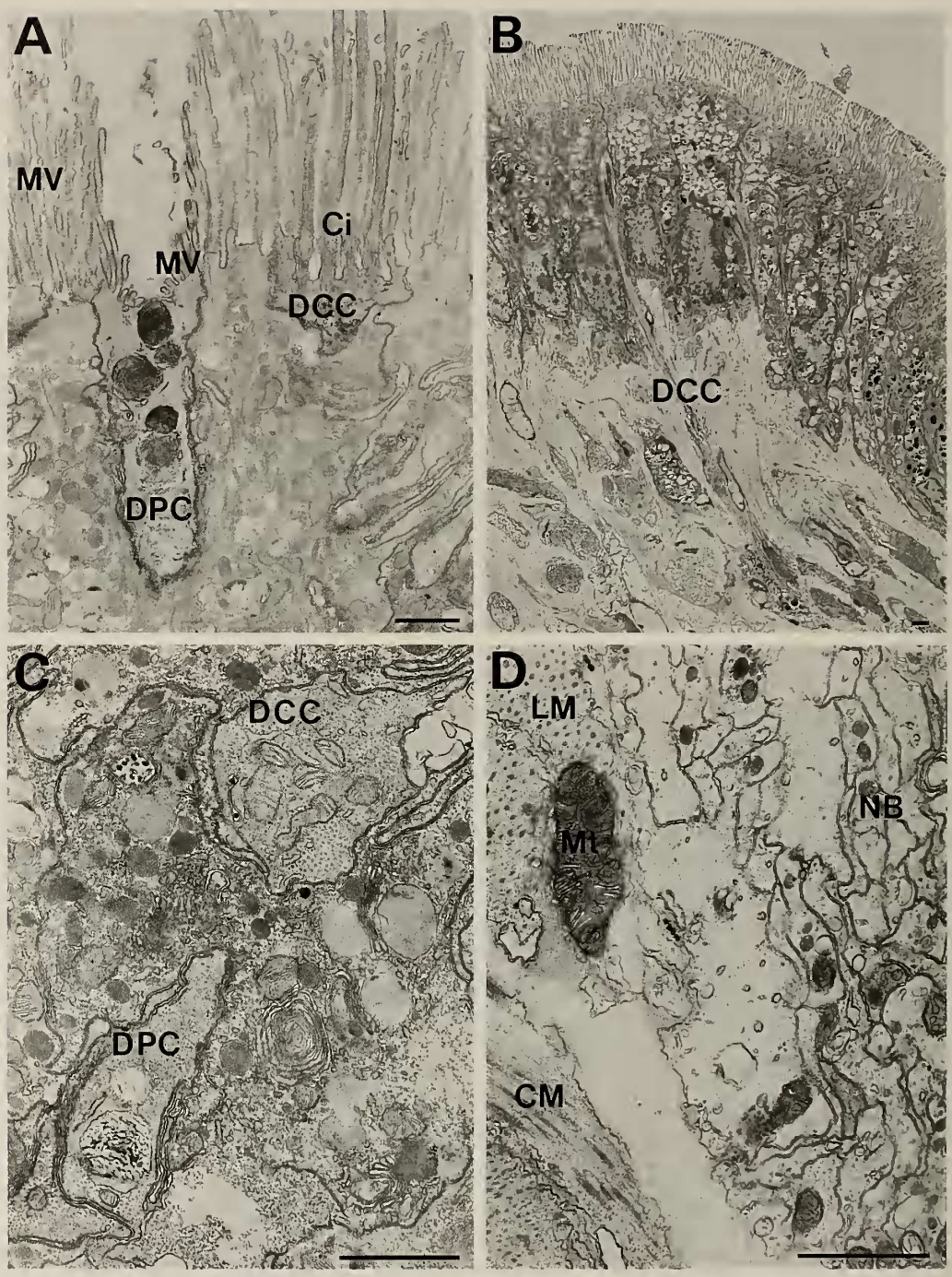

FIG. 5. Electron micrographs showing the distal processes of the ciliary cells (DCC) and pit cells (DPC), and nervous tissue. (A) Higher magnification showing cilia (Ci) of the ciliary cells, and a pit cell with its short microvilli (MV) in the outer epithelium of the siphon. (B) Lower magnification showing a fine distal process projecting from a ciliary cell. (C) A cross section of the distal processes of the ciliary and pit cells, showing many microtubules. (D) A cross section of the nerve bundle (NB) in the central region of the siphonal wall. CM, circular muscle; LM, longitudinal muscle. Scale bars, 1 μm.

DISCUSSION

It is unknown whether or not the mouse anti-squid rhodopsin serum used here cross-react with the *Ruditapes* rhodopsin, because a procedure to purify the *Ruditapes* rhodopsin has not yet been established. *Todarodes* and *Ruditapes*, however, belong to the same phylum, it is conceivable that their visual pigments will have sufficient homology for the anti-squid rhodopsin serum to cross-react with the *Ruditapes* rhodopsin.

The electron-microscopical observations revealed characteristic microvilli on the free surfaces of the outer and inner

EPCs (Fig. 4). It is suggested from the position of the microvilli (Figs. 2–4) and the appearance of the binding site of the anti-rhodopsin serum that the microvilli of the outer and inner EPCs contain rhodopsin-like immunoreactivity. The fact that the specific binding occurs in the overall surfaces of the outer and inner epithelia supports the idea above, because cells other than the EPCs, such as the ciliary and pit cells, are distributed only sparsely on both epithelia.

Intense fluorescence due to conjugated FITC was seen throughout the region beneath the inner epithelium (Fig. 2C). It is clear from electron-microscopical observations of the identical region (Fig. 4) that the fluorescence originates from groups of the granular cells. They can be seen scattered in the inner wall of the siphon with a low power dissecting microscope but their function remains to be investigated. In the SAB method, on the other and, the granular cells stained somewhat weakly with the serum (Fig. 3C, D). This inconsistency seems partly to be attributed to thickness of sections (15 μm in FITC, 5 μm in SAB). However, we should be cautious to decide whether or not the fluorescence of the granular cells is specific.

Although no differentiated photoreceptor cells were found in the siphon, *Ruditapes* clearly responds to illumination or shading of the siphon. The most probable candidate photoreceptor cells are the outer and inner EPCs which are provided with microvilli which show rhodopsin-like immunoreactivity. However, as has been known so far, photoreceptor cells which are able to transmit light information to secondary neurons and so on, and to induce effector responses are always nervous origin cells. Non-nervous cells such as epithelial cells have no axons and usually generate neither slow graded potentials nor action potentials. However, it has been reported in many hydroids (Cnidaria) that the epithelial cells generate electrically conductive action potentials which are able to excite nerves through electrical contact [13, 17]. Additionally, it has been reported in echinoids [21] and vertebrates [15] that pigment cells of non-nervous origin are directly photoresponsive. It may not therefore be unlikely that the outer and inner EPCs of *Ruditapes* siphon are photosensitive and pass light information to effector muscles through neihbouring nerves such as the ciliary and pit cells. Microvilli like those of the outer and inner EPCs are common in many molluscan epithelial cells (4, 11, 19, 20, 23) and their vertically projected appearance from the epithelial surface is reminiscent of those of primitive photoreceptor cells of some molluscs [3, 8, 9].

Crisp [5] has reported 2 types of sensory cells presumed to be mechanoreceptors cells (type I) and photoreceptor cells (type II) in the epithelium of a marine gastropod, *Nassarius*. The type I cells with cilia resemble the ciliary cells in *Ruditapes*. Type II cells with regressed cilia resemble the pit cells in *Ruditapes* except that the latter has no cilia. Based on morphology alone, the ciliary cells might be mechanoreceptive. The pit cells have short microvilli and a concaved distal portion, which rather suggest a chemoreceptive function. This does not necessarily mean that the ciliary and pit cells are not photosensitive, because directly photosensitive nerves are widely known in extraocular photoreceptive systems [18, 24].

Light [12] suggested from the light-microscopical observations of the siphon of *Mya*, that the pear-shaped cells may be photoreceptor cells. In *Ruditapes*, however, cells comparable to those of *Mya* could not be found, although there is presently no sufficient information on the ultrastructure of the pear-shaped cells.

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